

Roles of Endogenous Monocyte Chemoattractant Protein-1 in Ischemia-Induced Neovascularization

Hiroshi Niiyama, MD,* Hisashi Kai, MD, PhD,* Tomoka Yamamoto, BS,* Toshifumi Shimada, MD,* Ken-Ichiro Sasaki, MD, PhD,* Toyooki Murohara, MD, PhD,* Kensuke Egashira, MD, PhD,† Tsutomu Imaizumi, MD, PhD, FACC*

Kurume and Fukuoka, Japan

OBJECTIVES	We sought to investigate the role of endogenous monocyte chemoattractant protein (MCP)-1 in ischemia-induced neovascularization.
BACKGROUND	Roles of inflammatory changes including macrophage infiltration are suggested in ischemic neovascularization.
METHODS	Unilateral hindlimb ischemia was induced by excising surgically the entire femoral artery and vein in mice. Immediately after operation, plasmid deoxyribonucleic acid encoding a dominant negative mutant of MCP-1 (7ND) or the empty plasmid (mock) was injected into the ipsilateral thigh adductor muscle.
RESULTS	In mock-treated mice, MCP-1 was upregulated transiently in ischemic hindlimb peaking at day 3. Serial laser Doppler blood flow (LDBF) analysis showed an abrupt decrease in blood flow, followed by a recovery to the near-normal levels in mock-treated mice; 7ND treatment had no effects on the initial decrease in LDBF but deteriorated the recovery. At day 3, macrophage infiltration and inductions of tumor necrosis factor (TNF)-alpha and vascular endothelial growth factor (VEGF) were prominent in the ischemic adductor muscle in mock-treated mice; 7ND treatment significantly reduced macrophage infiltration and suppressed TNF-alpha and VEGF inductions in response to ischemia. At day 21, postmortem angiography and anti-CD31 immunohistostaining revealed well-developed collateral vessels and capillary formation, respectively, in the ischemic muscle of mock-treated mice; 7ND overexpression remarkably suppressed the collateral vessel formation and capillary formation.
CONCLUSIONS	Endogenous MCP-1 may play a role in ischemia-induced neovascularization by recruiting macrophages that activate TNF-alpha and VEGF inductions. (J Am Coll Cardiol 2004;44:661-6) © 2004 by the American College of Cardiology Foundation

The roles of macrophages have been highlighted in various cardiovascular diseases, including arteriosclerosis, atherosclerosis, and myocardial fibrosis (1-3). There is increasing evidence that inflammatory process is implicated as the trigger element of neovascularization in response to ischemia. Inflammatory cells, especially macrophages, infiltrate during the formations of capillaries (angiogenesis) and angiographically visible collateral vessels (arteriogenesis) in the ischemic tissue (4-7). Recently, we have shown that angiotensin II-mediated macrophage infiltration precedes neovascularization in unilateral ischemic hindlimb model, and the infiltrated macrophages produce vascular endothelial growth factor (VEGF) in response to ischemia (8). Also, Arras et al. (4) revealed that macrophages are the main source of tumor necrosis factor (TNF)-alpha during collateral vessel formation in the rabbit ischemic hindlimb. In addition, earlier studies demonstrated that exogenous administration of monocyte chemoattractant protein-1

(MCP-1), a key molecule for the recruitment of monocytes/macrophages (9), accelerated ischemia-induced formation of collateral vessels in the rabbit hindlimb (7,10). Taken together, the possible role of MCP-1 was suggested in ischemia-induced neovascularization by recruiting macrophages that, in turn, produce angiogenic growth factors such as VEGF and TNF-alpha. However, there are no available data regarding the specific role of endogenous MCP-1 in ischemia-induced arteriogenesis and angiogenesis.

A mutant of MCP-1, which lacks the N-terminal amino acids 2 to 8 (7ND), has a potent dominant negative activity (11). A series of recent studies have shown that overexpression of 7ND is a useful strategy for blocking MCP-1 activity in vivo in various cardiovascular disease models (12,13). In the present study, we sought to determine the role of the endogenous MCP-1 in ischemia-induced neovascularization in mouse hindlimb. For this purpose, effects of blocking MCP-1 activity in the ischemic hindlimb were investigated by overexpressing 7ND in the ischemic tissue.

METHODS

Human 7ND complementary deoxyribonucleic acid (DNA) was constructed by recombinant polymerase chain reaction using a wild type MCP-1 complementary DNA and cloned into Bam HI (5') and Not I (3') site of the pCDNA3 expression plasmid vector (Invitrogen Corp., Tokyo, Japan),

From the *Third Department of Internal Medicine and Cardiovascular Research Institute, Kurume University, Kurume, Japan; and †Cardiovascular Medicine, Kyushu University Graduate School of Medicine, Fukuoka, Japan. Supported, in part, by a grant for Science Frontier Research Promotion Centers and Grants-in-Aid for Scientific Research (Drs. Kai and Imaizumi) from the Ministry of Education, Science, Sports and Culture, Japan and a grant from the Ministry of Health, Welfare, and Labor, Japan (Dr. Imaizumi).

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Abbreviations and Acronyms

DNA	= deoxyribonucleic acid
EC	= endothelial cell
LDBF	= laser Doppler blood flow
MCP-1	= monocyte chemoattractant protein-1
TNF	= tumor necrosis factor
VEGF	= vascular endothelial growth factor
7ND	= a dominant negative mutant of MCP-1 lacking the N-terminal amino acids 2 to 8

as described previously (12). Twenty-four nucleotides encoding FLAG epitope were added directly at the 3' terminus of 7ND sequence (12). All sequences were confirmed by double-stranded DNA sequencing. The dominant negative activity was verified in vivo as described previously (12).

Animal model and gene transfer. The study protocol was approved by the Institutional Animal Care and Treatment Committee. After being anesthetized with intraperitoneal pentobarbital (50 mg/kg), the entire left femoral artery and vein were excised surgically in eight-week-old male C57BL/6 mice (Clea Japan Inc., Tokyo, Japan), as described previously (14,15). Immediately after operation, 30 μ l of saline containing denoted dose of plasmid encoding 7ND complementary DNA or the empty plasmid (mock) was injected into the exposed ipsilateral thigh adductor muscle. Sham mice underwent the same operational procedure except that the artery and vein were left intact.

Expression of transfected 7ND was determined in the thigh adductor muscle on the basis of Western blot analysis using an anti-FLAG antibody. After mice were killed by an overdose of intraperitoneal pentobarbital, the thigh adductor muscle was removed, snap-frozen in liquid nitrogen, and stored at -80°C until use. The tissue was homogenized by using FastPrep homogenizer (ThermoSavant, Holbrook, New York), and the tissue protein was extracted, separated on 10% SDS-PAGE, and subjected to Western blot analysis (16). As shown in Figure 1, 7ND expression was transiently observed in the adductor muscle, peaking at days 3 to 7 and returning to insignificant levels by day 14 after operation and gene transfer.

Laser Doppler blood flow (LDBF) analysis. Hindlimb blood flow was evaluated serially in each mouse ($n = 12$ per group) using a LDBF image analyzer (Moor Instruments, Devon, United Kingdom) before surgery and at postopera-

tive days 3, 7, 14, 21, 28, and 35, as previously described (15). After scanning twice, the average LDBF values in the ischemic and normal (nonischemic) legs were calculated on the basis of colored histogram pixels. To avoid data variations raised from ambient light and temperature, relative changes in hindlimb blood flow were expressed as the ratio of the left (ischemic) over right (normal) LDBF.

Histologic analysis. After mice were killed with an overdose of pentobarbital, the thigh adductor muscle was excised, fixed in methanol, embedded in paraffin, and then sectioned into 5- μ m slices.

CAPILLARY DENSITY. Capillary endothelial cells (ECs) were identified by immunohistostaining with an anti-CD31 monoclonal antibody (Pharmingen, Franklin Lakes, New Jersey). The number of capillaries was counted and averaged in 15 random microscopic fields from three independent cross-sections of the adductor muscle in each animal ($n = 12$) at day 21, and then capillary density was expressed as the number of capillaries per high-power field ($\times 400$).

MACROPHAGE DENSITY. Infiltrated macrophages were detected immunohistochemically by using an anti-F4/80 monoclonal antibody (Serotec, Raleigh, North Carolina) in the cross-section of the adductor muscle at day 3. Macrophages were counted in 15 random microscopic fields from three independent sections of each animal ($n = 8$), and macrophage density was described as the number of F4/80-labeled cells per high-power field ($\times 400$) (8).

Tissue contents of MCP-1, VEGF, and TNF-alpha. At denoted day, the thigh adductor muscle ($n = 8$ per group) was excised, snap-frozen in liquid nitrogen, and stored at -80°C until use. After homogenization by using FastPrep (ThermoSavant), the tissue proteins were extracted, and the contents of MCP-1, VEGF, and TNF-alpha were determined by using a commercially available ELISA kit for mouse MCP-1, mouse VEGF, and mouse TNF-alpha (R&D System, Minneapolis, Minnesota), respectively, according to the manufacturer's instruction.

Postmortem angiography. At day 21, mice ($n = 12$ per group) were killed by injecting intraperitoneally an overdose of pentobarbital. Postmortem angiography was performed by injecting 200 μ l of contrast media through a 26-gauge soft-tip catheter, which was inserted into the abdominal aorta, at a perfusion pressure of 80 to 90 mm Hg. X-ray angiograms were taken using a mammography system (Senographe 500T, GE Medical Systems, Tokyo, Japan), and the extent of collateral vessel formation was quantified by angiographic score, as described previously (8,17).

Statistical analysis. Statistical analysis was adequately performed by unpaired Student *t* test or analysis of variance followed by Scheffé's *F* test. A value of $p < 0.05$ was considered statistically significant. Angiographic score, capillary density, and macrophage count were evaluated by two observers in a blind manner, and the values obtained by the two observers were averaged. The interobserver or intraobserver variation was $<5\%$ in each experiment.

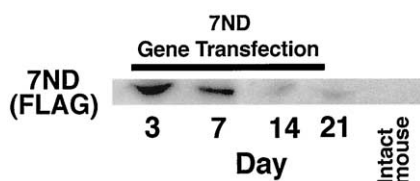


Figure 1. Expression of transfected 7ND in the ischemic adductor muscle. Western blot analysis using an anti-FLAG M2 monoclonal antibody showed a transient expression of FLAG-fusion 7ND protein in the muscle. A total of 50 μ g of the total protein was added in each lane. The similar results were obtained from three independent experiments.

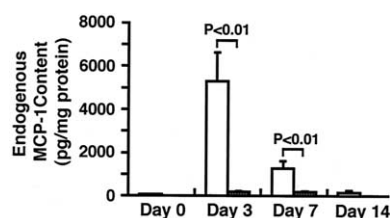


Figure 2. Endogenous monocyte chemoattractant protein-1 (MCP-1) induction in the ischemic hindlimb. Pooled data of temporal changes in endogenous MCP-1 content in the thigh adductor muscle in the ischemic hindlimb (open bars). Solid bars = sham mice. Bar = $1 \times \text{SD}$ ($n = 8$).

RESULTS

Irrespective of 7ND gene transfer, all mice survived after surgery of unilateral hindlimb ischemia and looked healthy during the observation period. Body weight was similar among the study groups over the time course. Also, there was no significant difference in blood pressure among 7ND-treated (up to 300 μg) and mock-treated mice (data not shown).

Transient MCP-1 expression in ischemic hindlimb. To determine the change in endogenous MCP-1 expression, tissue content of MCP-1 was measured in the thigh adductor muscle. Tissue MCP-1 levels were quite low in sham mice during the observation period (data not shown). Endogenous MCP-1 was transiently and remarkably up-regulated in the ischemic muscle with a peak at day 3, remaining relatively high at day 7, and returning to the baseline by day 14 (Fig. 2).

Deteriorated recovery of LDBF of ischemic hindlimb by 7ND overexpression. In mock-treated mice, serial LDBF analysis demonstrated an abrupt decrease in blood flow of

the left ischemic hindlimb, followed by a gradual recovery to a near-normal level by day 35, whereas there were no changes in blood flow of the right normal hindlimb (Fig. 3). In mice with 7ND gene transfer, the severity of the induced ischemia was similar to that in mock-treated mice. However, 7ND overexpression deteriorated the recovery of the hindlimb blood flow during the observation period, and the ratio of ischemic/normal LDBF was persistently lower in 7ND-treated mice compared with mock-treated mice. The effects of 7ND were dose-dependent on the amount of the injected 7ND plasmid, and 200 μg of 7ND plasmid was the minimum dose that induced the maximum inhibitory effect (Fig. 3B). Accordingly, 200 μg of 7ND was used in the following experiments.

Effects of 7ND on macrophage infiltration. Inflammatory cell infiltration and skeletal muscle degeneration were observed in the ischemic adductor muscle of mock-treated mice at day 3 (Fig. 4A). It was apparent that less infiltrated cells were found in 7ND-treated mice although the extent of skeletal muscle degeneration was not affected by 7ND overexpression. The number of F8/40-positive macrophages were examined at day 3 when a transient infiltration of macrophages peaked (8). Macrophage infiltration was remarkably reduced by 7ND overexpression to 20% of mock-treated mice (Fig. 4B). At day 21, irrespective of 7ND treatment, regeneration of skeletal myocytes was evident, and the infiltrated cells were scarcely found in ischemic muscle.

Reduced expression of angiogenic factors in 7ND-treated mice. Ischemia transiently enhanced VEGF expression in the adductor muscle with a peak at day 3 (Fig. 5). At days 3 and 7, 7ND overexpression significantly

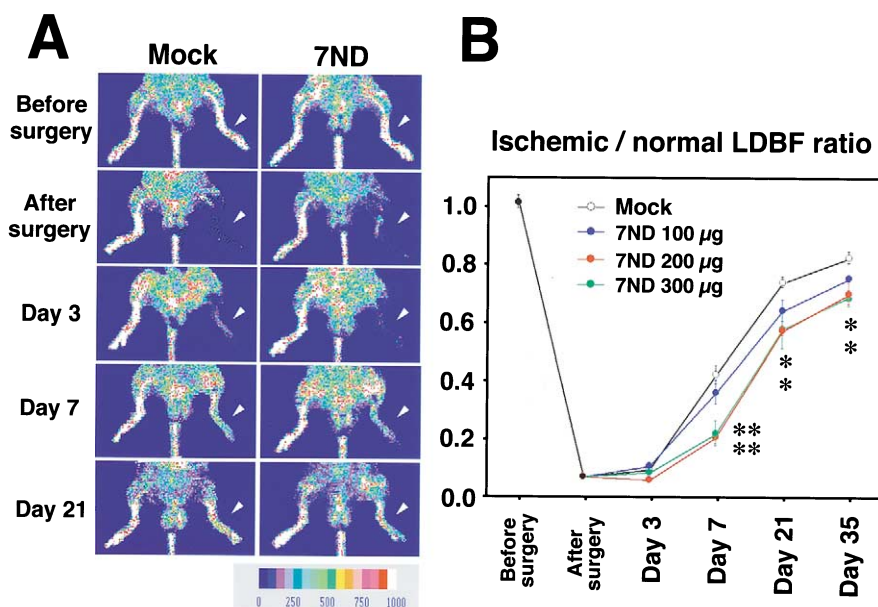


Figure 3. Laser Doppler blood flow (LDBF) analysis was performed serially in each mouse to determine the blood flow in unilateral ischemic hindlimb. (A) Representative LDBF images of the ischemic (arrow heads) and nonischemic hindlimb in 7ND-transfected and mock-treated mice. (B) Pooled data of temporal changes in ischemic/nonischemic LDBF ratio. The recovery of LDBF ratio was deteriorated by 7ND overexpression in a dose-dependent manner. * $p < 0.05$ and ** $p < 0.01$ vs. mock-treated mice. Bar = $1 \times \text{SD}$ ($n = 12$).

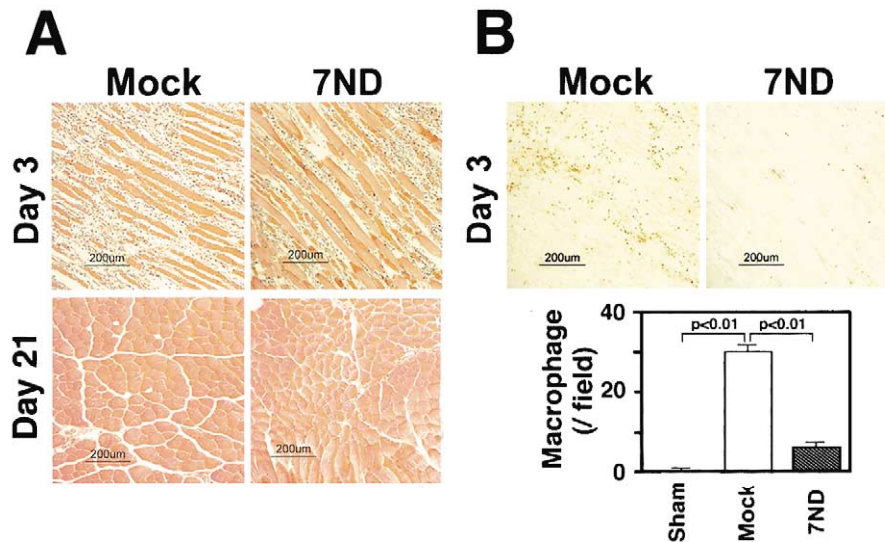


Figure 4. (A) Representative microphotographs of hematoxylin-eosin-stained sections of ischemic thigh adductor muscle obtained from mock- and 7ND-treated mice at days 3 and 14. (B, top) Representative immunohistostainings for F4/80, a macrophage-specific marker, of the adductor muscles at day 3. The serial sections were subjected to the experiments for A (day 3) and B. (B, bottom) Pooled data showing the number of infiltrated macrophages in mock- and 7ND-transfected mice at day 3. Bar = $1 \times \text{SD}$ ($n = 8$).

decreased the ischemia-induced VEGF induction to the levels, which were still significantly higher than that of sham mice.

Tumor necrosis factor- α is known as not only an inflammatory mediator but also a potent inducer of the ischemia-induced arteriogenesis (7). In mock-treated mice, TNF- α expression was transiently induced in the adductor muscle, peaking at day 3. The ischemia-induced TNF- α upregulation was remarkably inhibited by 7ND overexpression at day 3, declining to the sham level at day 7.

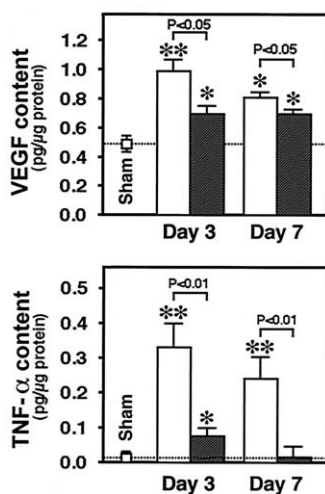


Figure 5. Pooled data demonstrating the effects of 7ND gene transfer on vascular endothelial growth factor (VEGF) induction and tumor necrosis factor (TNF)- α induction in ischemic thigh adductor muscle at days 3 and 7. Bar = $1 \times \text{SD}$ ($n = 8$). Solid bars = 7ND-transfected mice; open bars = mock-treated mice; open squares = sham-operated mice without any gene transfection. * $p < 0.05$ and ** $p < 0.01$ versus sham mice. Analysis of variance followed by Scheffé's F test was performed for statistical comparison.

Impaired neovascularization in 7ND-treated mice. The development of angiographically visible collateral vessels and capillary formation were studied to determine whether impaired neovascularization was associated with reduced LDBF in ischemic hindlimb treated with 7ND. First, at day 21, postmortem angiography was performed, and the angiographic score was investigated (Fig. 6A). Mock-treated mice showed well-developed collateral vessels in the ischemic hindlimb. The number of collateral vessels was significantly reduced by overexpressing 7ND, and 7ND decreased the angiographic score to 60% of mock-treated mice.

Next, capillary density was evaluated for specific evidence of neovascularization at the microcirculation level (Fig. 6B). In mock-treated mice, a number of the capillaries surrounded by CD31-positive ECs were found in the adductor muscle of the ischemic hindlimb at day 21. Overexpression of 7ND significantly reduced the capillary density in the ischemic muscle to 35% of mock-treated mice. There was no spatial variation of the distribution of the capillaries in the ischemic adductor muscles in mock- and 7ND-treated mice.

DISCUSSION

The present study demonstrated that MCP-1 is transiently induced in the ischemic hindlimb of mice, which is the well-studied model of ischemia-induced neovascularization (14). Blockade of endogenous MCP-1 activity by 7ND overexpression remarkably inhibited ischemia-induced macrophage infiltration and significantly reduced VEGF and TNF- α inductions in the ischemic tissue in the early phase. Furthermore, 7ND overexpression significantly impaired the recovery of the LDBF reduction associated with

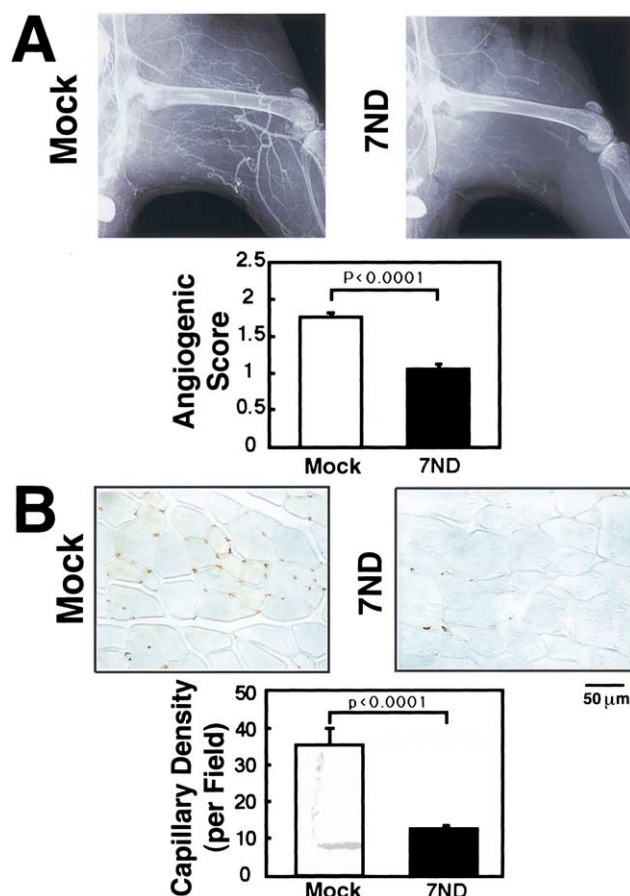


Figure 6. Effects of 7ND overexpression on ischemia-induced neovascularization. (A) Postmortem angiograms at day 21; 7ND overexpression eliminated the development of angiographically visible collateral vessels (top), and angiographic score was significantly lower in 7ND-treated mice as compared with mock-transfected mice (bottom). Bar = $1 \times \text{SD}$ ($n = 12$). (B) Capillary density of the thigh adductor muscle at day 21. Immunohistostaining for CD31, an endothelial cell marker (stained as brown), was performed to determine the capillaries in the ischemic muscle (top). Capillary density at day 21 was significantly reduced by 7ND gene transfer in the ischemic hindlimb (bottom). Bar = $1 \times \text{SD}$ ($n = 12$).

reduced angiographically visible collateral and capillary formations in the ischemic hindlimb in the late phase.

As shown in Figure 2, unilateral hindlimb ischemia induced a transient and remarkable MCP-1 induction in the thigh adductor muscle, peaking at day 3. It was noteworthy that the peak of MCP-1 induction coincided with that of macrophage infiltration seen in our previous study (8). Thus, we sought to clarify the role of endogenous MCP-1 by overexpressing 7ND, a dominant negative mutant of MCP-1, in the ischemic adductor muscle, because this mutant can bind to the MCP-1 receptors on the target cells but does not induce the intracellular signaling activation (11). When complementary DNA encoding 7ND was injected into the adductor muscle at the time of the surgical removal of femoral artery and vein, a transient, yet significant, expression of 7ND protein was observed on the basis of Western blotting against the FLAG epitope tag (Fig. 1). Because transfected 7ND was expressed as long as ischemia-induced MCP-1 induction (Fig. 2) was present, it was expected that activity of endogenous MCP-1

would be blocked in ischemic hindlimb. Indeed, as shown in Figure 4, macrophage infiltration was remarkably inhibited in the ischemic muscle by 7ND overexpression. This finding indicates that MCP-1 is a major determinant of the macrophage recruitment into the ischemic hindlimb. Along with ischemic hindlimb, MCP-1 induction was documented in ischemic cerebral cortex (18), ischemia-reperfused heart (19), ischemia-reperfused kidney (20), and ischemic retinopathy (21). The precise mechanism whereby ischemia induces MCP-1 induction remains unknown at present. Activation of local angiotensin II system may be involved in one of the mechanisms, because MCP-1 induction in the ischemic hindlimb is remarkably attenuated in the angiotensin II type 1 receptor knockout mice (8).

The present study demonstrated that when MCP-1 activity was blocked, inductions of VEGF and TNF- α were significantly reduced in the ischemic hindlimb (Fig. 5). The attenuation of VEGF and TNF- α inductions could be responsible for the decreased formation of capillaries and angiographically visible collaterals, resulting in deteriorated recovery of blood flow of the ischemic hindlimb in 7ND-treated mice. It is indicated that infiltrated macrophages may facilitate ischemia-induced arteriogenesis and angiogenesis by producing VEGF (8). Moreover, activated macrophages have been shown to predominantly produce TNF- α upon the hindlimb ischemia (4). Tumor necrosis factor- α is a major mediator of inflammatory reactions, and also participates in arteriogenesis characterized by the formation of angiographically visible collaterals in the ischemic hindlimb (4). Because TNF- α is responsible for adhesion and activation of additional macrophages via upregulation of cell adhesion molecules on both ECs and macrophages (22,23), the angiogenic properties of TNF- α may be attributable, in part, to the formation of positive feedback loop of the macrophage-mediated mechanisms. Taken together, inhibition of TNF- α induction may lead to the elimination of ischemia-induced neovascularization both through the prevention of TNF- α -mediated exaggeration of inflammatory process and through the attenuation of the angiogenic properties of TNF- α . In addition, it has been shown that ECs express the MCP-1 receptor CCR2 and that MCP-1 induces proliferation and migration of ECs (24). Thus, it is also possible that 7ND overexpression may inhibit the direct angiogenic effect of MCP-1. As shown in Figures 4B and 5, the inhibitory effect of 7ND on VEGF induction was smaller than those on macrophage infiltration. Our previous study demonstrated that, in addition to inflammatory cells, skeletal myocytes express VEGF in the adductor muscle of the ischemic hindlimb (8). Thus, the skeletal myocytes, which were not inhibited by 7ND, may be responsible for the 7ND-insensitive VEGF production.

It was apparent that the observed effects of 7ND are partial on the inhibition of ischemia-induced neovascularization, although macrophage infiltration was almost abolished. The reduction in LDBF ratio by 7ND was most evident at day 7,

and thereafter the extent of the LDBF reduction was smaller in the later phase. It was possible that inflammatory process was activated after 7ND expression had decayed. However, this does not seem the case because there were only sparse interstitial cells in the ischemic thigh muscle after day 14, irrespectively of 7ND treatment (Fig. 4). Another possible explanation is that macrophage-mediated inflammation participates in the recovery of LDBF of the ischemic hindlimb, mainly in the early phase, as an initiator of ischemic neovascularization. Thus, the 7ND-insensitive neovascularization, especially in the later phase, may be dependent upon the mechanisms related to cells other than macrophages.

At day 21, 7ND overexpression remarkably reduced collateral vessel formation and the intramuscular capillary formation in the ischemic hindlimb, whereas the inhibitory effects on the recovery of LDBF were modest. In the present study, we used an LDBF imaging system that can detect blood flow of the tissue surface to a depth of 600 μm (14), indicating that LDBF images reflect mainly the superficial and subdermal blood flow and, to lesser extent, the intramural blood flow. This may explain the difference in the response to 7ND between LDBF image and other parameters of neovascularization (angiography and histology). This issue should be addressed in future studies.

Study limitations. It has been shown that 7ND is not only expressed in the transfected site (e.g., thigh muscle) but also is secreted into the systemic circulation and, in turn, blocks MCP-1 activity in the remote organ (12,13). Thus, we do not exclude the possibility that mobilization of endothelial progenitor cells from bone marrow is reduced by 7ND overexpression in ischemic hindlimb.

In conclusion, neovascularization and blood flow recovery in response to hindlimb ischemia were significantly impaired in mice with functional blockade of MCP-1 activity. Thus, it is indicated that the induction of endogenous MCP-1 participates in angiogenesis and arteriogenesis by recruiting macrophages into the ischemic tissue. The present study would provide an insight into better understanding of the underlying mechanism of ischemia-induced neovascularization.

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Reprint requests and correspondence: Dr. Hisashi Kai, Third Department of Internal Medicine and Cardiovascular Research Institute, Kurume University, 67 Asahi-machi, Kurume 830-0011, Japan. E-mail: naikai@med.kurume-u.ac.jp

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